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Malignant Melanoma – Future Prospects

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SUMMARY As the incidence and already high mortality rates of malignant melanoma have been steadily increasing in recent decades, the early detection and excision of malignant melanoma have imposed as the most important task. Staging of malignant melanoma is determined according to the level of invasion (Clark level) and vertical thickness (Breslow scale). Besides operative therapy, which is the only effective treatment for malignant melanoma, postoperative adjuvant chemotherapy, immunotherapy, radiotherapy, and biologic therapy also are of great importance. In recent years, immunologic strategies including tumor vaccine and adjuvant therapy with interferon-alfa have been attempted to improve survival of patients with more advanced malignant melanoma. A recent melanoma research has focused on target therapy such as immunotherapy (vaccines, monoclonal antibodies, dendritic cells) and gene therapy. Genetic immunization has become an attractive strategy for the development of melanoma vaccines, because a number of antigens recognized by cellular components of the immune system have been identified at the molecular level. Numerous chemotherapeutic agents have shown activity in the treatment of metastatic malignant melanoma, such as dacarbazine (dimethyl triazene imidazole carboxamide); other agents have been used, however, with less success. However, a very modest effect was recorded in advanced malignant melanoma. There are many experimental trials using combined therapy for malignant melanoma, including chemotherapy (dimethyl triazene imidazole carboxamide) and biologic therapy (interleukin (IL)-2, interferon (IFN)-gamma, IFN-alfa). The results obtained open particularly interesting prospects in the field of malignant melanoma with high relevance for its development and progression. Molecular therapeutics and vaccine development will probably be an important focus for the future melanoma treatment.

KEY WORDS: malignant melanoma; immunotherapy; gene therapy; chemotherapy

INTRODUCTION

Cutaneous melanoma is a tumor resulting from complex interactions between genetic, constitutional and environmental factors (1-3). Its incidence and already high mortality rates have been steadily increasing over recent decades. The risk factors associated with melanoma include fair complexion, a history of multiple childhood blistering sunburns, an increased number of common acquired and dysplastic nevi, changes in a nevus, and family history of melanoma. Malignant melanoma (MM) is a skin tumor fatal for the majority of patients, and early detection is of high importance. This is supported by the fact that individuals with cutaneous melanoma have a higher survival rate

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in developed countries, indicating that public education about MM results in earlier diagnosis, treatment and potential cure.

DIAGNOSTIC TECHNIQUES FOR MALIGNANT MELANOMA

Different modalities are used to identify MM in clinical practice: ABCD method, total-body photography, skin surface microscopy, and machine dermatoscopy vision. Staging of MM is determined according to the level of invasion (Clark level) and vertical thickness (Breslow scale) (1-3). Clark's method of microstaging is based on five levels of tumor invasion into the dermis and subcutaneous fat, while Breslow's method is based on vertical thickness (in millimeters) of the primary tumor from the epidermal granular layer (or the base of the lesion if the tumor is ulcerated) to the deepest identifiable contiguous melanoma cell (1-3). The American Joint Committee on Cancer (AJCC) has developed a staging system incorporating Breslow tumor thickness, Clark levels, and various other tumor characteristics. In 1969, clinicians and scientists from various disciplines and fields of research with a common interest in MM formed the EORTC Melanoma Group (MG), which has pursued a scientific approach to the development of treatment for MM (4).

A systematic review of the literature reveals various diagnostic techniques for melanoma including naked-eye clinical examination, clinical examination with the aid of total-body photographs, epiluminescence microscopy (ELM), digital ELM, computer-assisted techniques, near-infrared applications, teledermatology, etc. (1-3). Once a melanoma has been clinically identified and excised with adequate margins, it is important to detect any remaining or recurring tumor cells. One of the molecular diagnostic methods used for this purpose is reverse transcriptase polymerase chain reaction (RT-PCR), the most reliable and reproducible method for the detection of low-level disease. Therefore, RT-PCR may increase the power of minimal residual disease and micrometastatic disease detection in some patients, resulting in more accurate prognosis and/or improvement in treatment timing or selection. Nucleic acid sequence-based amplification and RT-PCR both successfully amplified target RNA in peripheral blood samples from patients with MM, although its utility may be limited by the lack of automation and reproducibility. A novel imaging technique, near-infrared technique for *in vivo* microscopic analysis of skin lesions including pigmented lesions, may aid in the early detection of clinically barely visible or nonpigmented melanomas and may facilitate preoperative noninvasive assessment of their margins (2,5). However, the rapid development of melanoma research produces new diagnostic techniques as well as potential new therapies.

EXPLORATION OF MODERN THERAPEUTIC METHODS FOR MALIGNANT MELANOMA

Besides surgical therapy, which is the only effective treatment for MM, postoperative adjuvant chemotherapy, immunotherapy, radiotherapy, and biologic therapy are also important. Adjuvant interferon-alfa-2b and various experimental melanoma vaccines are the most recent options that are currently being studied. Recent melanoma research has focused on target therapy such as immunotherapy (vaccines, monoclonal antibodies, dendritic cells) and gene therapy, with the task to decrease their toxicity and enhance specific cytotoxicity.

IMMUNOTHERAPY

Different types of immunotherapy for MM are used, as yet only in the experimental phase, and the aim is to ensure a safe and efficient type of adjuvant therapy (Tables 1 and 2) (6). Nonspecific immunotherapy stimulates the reticuloendothelial system, whereas active specific immunization is considered to induce host response to tumor-as-

Table 1. Treatment for malignant melanoma according to stages

Therapy for primary melanoma:
- complete surgical excision of primary lesion
- elective lymph node dissection
- sentinel lymph node biopsy

Therapy for regional metastases:
- surgery
- isolated limb perfusion
- adjuvant therapy (radiotherapy, chemotherapy, regional limb perfusion, IFN-alfa)

Therapy for distant metastases:
- surgery
- radiotherapy
- chemotherapy (single-agent or combination chemotherapy)
- chemoimmunotherapy (IFN-alfa + chemotherapy)
- biologic therapy (IFN-alfa, IL-2, IL-2+LAK cells, monoclonal antibodies, melanoma vaccines)

Table 2. Immunotherapeutic strategies for malignant melanoma

IFN:
IFN-alfa as single agent or in combination with chemotherapy

Interleukins:
IL-2
IL-2 plus LAK cells
IL-2 plus IFN

Monoclonal antibodies:
MAbs against melanoma-associated Ags (peptidoglycans, transferrin receptor-related Ags, glycoproteins, high-molecular melanoma-associated Ags, sialoglycoproteins, gangliosides)

Melanoma vaccines:
- polyvalent vaccine with whole melanoma cells (e.g., autologous - autologous vaccines + haptens /DNP, BCG/ or cytokines /IL-2, GM-CSF/)
- allogeneic vaccine (e.g., polyvalent melanoma cell vaccine: PMCV/Cancer-Vex Melacine)
  - cell lysate (e.g., vaccinia melanoma cell lysate (VMCL))
  - partially purified Ags (e.g., shed-Ag vaccine and mechanical cell lysates)
  - univalent vaccine: purified Ags (e.g., GM2-KLH/QS - 21); peptide
  - Ags (e.g., tyrosinase, gp-100)

sociated antigens (Ag). Passive immunotherapy implies the introduction of *in vitro* activated antitumor effector cells and includes two modalities of the use of gene transfer techniques: a genetic modification of effector cells and amplification of pre-effector cells. The effects of recombinant adenovirus and vaccinia virus vectors have been investigated (3). A new, polyvalent vaccine produced from allogeneic tumor inoculated with tumor-cell lysing vaccinia has been used. The administration of the cell lysate i.i. together with DETOX adjuvant (detoxicated endotoxin of cellular cytoskeleton), squalene oil, and emulsifier provoked an initial response in 50% of patients (7). Another vaccine composed of allogeneic tumor cells and BCG resulted in better survival compared with control group, especially in men younger than 57 and with less than five positive lymph nodes (8). The interest in specific active immunotherapy arose about 40 years ago due to a case of MM, which had regressed spontaneously after a blood transfusion. MM-associated antigens and antibodies (Abs) reacting with melanoma cell membrane Ags have been identified in MM patients. One type of specific active immunotherapy are melanoma vaccines based on the melanoma cell-expression of human leukocyte antigens (HLA) and tumor-associated Ags. MM vaccines such as purified gangliosides, shed antigens, mechanical or viral lysates, and allogeneic and autologous preparations of whole cells, mostly cytokines, have been tried to induce or enhance immunity against MM-associated Ags. Multiple tumor Ags have been identified although little is known about which one would be most effective in cancer immunotherapy (9). To get an insight into the selection of appropriate Ags, some authors measured immune reactivity of patients who had complete regression of melanoma metastases. Studies revealed a melanoma Ag, identified as the BING-4 protein, encoded in a gene-rich region of the extended class II MHC, as an antigen with a potential use in immunotherapy of patients with MM (9). There also are other potential vaccine components such as gangliosides, e.g., GD3, GM2 and GD2, that are abundantly expressed on the surface of malignant cell, suggesting the potential anti-ganglioside antibody therapy (10). Koh *et al*. have reported on high *in vivo* antitumor effects of anti-GM2 ganglioside Abs against tumor cells, which is currently used in clinical trials for melanoma (10). Based on the expression of N-glycolyl-containing gangliosides and Ags on human melanoma cells, 1E10 anti-Id vaccine was used for the treatment of MM (11). The generation of 1E10 [gamma]-type anti-idiotype mAb (Ab2) specific to an Ab1 mAb, which is able to react specifically with the cells, performed in a clinical trial in 20 patients with advanced MM, revealed the 1E10 anti-Id vaccine to be a safe, well tolerated, and immunologically effective method (11).

Different antigens may be used as melanoma vaccine candidates alone or in combination with other tumor antigens, e.g., decapeptide ELA (ELAGIGILTV). The recombinant outer membrane protein A of *Klebsiella pneumoniae* (kpOmpA), P40, was recently shown to target dendritic cells and to induce peptide-specific CTLs, suggesting the adjuvant role of P40 mixed or chemically conjugated to ELA, and P40/ELA as an important melanoma vaccine candidate (12).

Identification of tumor antigens and their optimal antigenic peptides has raised hope for the development of peptide-based immunotherapeutic vaccine strategies for human melanoma. However, synthetic peptides alone are not immunogenic enough and an appropriate formulation is critical for the elaboration of peptide vaccines.

The results reported from a study indicated that a single palmitoyl-lysine chain is enough to assure immunogenicity of a given peptide. The presence of a lipid tail bypasses the need of an adjuvant. Identification of Melan-A/MART-1 antigen immunodominant peptide analog supported the selection of MART-lipopeptide melanoma vaccine for evaluation in a clinical trial (13).

Adoptive immunotherapy implies passive transfer of activated immune cells to the tumor patient in order to stimulate tumor regression, e.g., lymphoid cells, IL-2, tumor infiltrating lymphocytes (TIL), lymphoid cells from regional lymph nodes, and T-cells that are immunospecific for reactivity to MM (14). Lymphoid cells recognize antigen and produce cytokines (TNF and IFN-gamma, GM-SCF), which can directly destroy the tumor and attract other host cells to mediate tumor regression. An example of such therapy is the use of IL-2 (acts as a growth factor for the cells) with infusion of lymphoid activated cells forming so-called lymphokine-activated killer (LAK) cells, with response in 15% of patients (15).

GENE THERAPY

Gene therapy is a new therapeutic method in the treatment of MM, which attempts to transform genetic material into human cells. Various gene therapy strategies for MM are being evaluated in multiple clinical trials all over the world. There are gene modified cancer vaccines (GMTV) modified with genes encoding cytokines or costimulatory molecules and dendritic cells modified with genes encoding tumor antigens or immunostimulatory factors (16).

The results reported from a study indicate that two genes are implicated in the pathogenesis of melanoma, i.e. CDKN2A and CDK4. The CDKN2A gene is a tumor suppressor gene with germline mutations detected in 20% of melanoma-prone families, and is an appealing candidate for MM susceptibility (17). The CDK4 gene is an oncogene with cosegregating germline mutations detected in only three kindreds worldwide. Thus, the phenotype observed in melanoma-prone CDK4 families appears to be more complex than just the CDK4 mutation. Although CDK4 is a melanoma susceptibility gene, it plays a minor role in hereditary melanoma. Suicide gene therapy in combination with prodrugs represents an attractive approach to the treatment of cancer but it is limited by the difficulty in targeting tumor cells, especially those at distant metastases associated with the most complex tumors (18). For this reason, attempts to stimulate global anti-tumor immune responses at the sites of effective suicide gene/prodrug-mediated tumor cell destruction are appealing. It included a gene coding for secreted secondary lymphoid tissue chemokine (SLC) along with the herpes simplex virus thymide kinase (HSV-TK) gene, and a bicistronic vector for anti-tumor gene therapy in conjunction with the prodrug ganciclovir (GCV). They mediated a greatly enhanced anti-tumor effect in the murine B16 melanoma tumor model and the obtained enhanced antitumor effect was the result of a strong induced cytotoxic T-lymphocyte (CTL) immune response resulting from the recruitment of immune cells to the site of HSV-TK/GCV-induced tumor destruction by the potent chemokine SLC (18).

Attempts have been made to transfer genetically modified TIL cells secreting TNF that selectively migrates to the tumor site, and tumor transfer employing an *in vivo* model, i.e. by CT-guided intratumor DNA inoculation associated with liposomes was also done (19).

Certain genes may suppress growth and induce apoptosis in a variety of human tumor cell lines. Subtraction hybridization identified mda-7, a melanoma differentiation associated (mda) gene, as a negative regulator of its growth and progression. Mda-7 is a ubiquitous inhibitor of growth in diverse human cancers and may have direct translational potential for gene-based therapy (20). Thus, ectopic transfer of mda-7 *in vitro* suppressed growth and induced apoptosis in a variety of human tumor cell lines but not normal cells (21). The expression of mda-7 inversely correlates with melanoma development and progression, and increasingly reduces expression in radial growth phase, vertical growth phase and metastatic melanoma.

After treatment with a combination of fibroblast interferon (IFN-beta) and the protein kinase C activator mezerein (MEZ), the human melanoma cell growth is arrested irreversibly, they lose their tumorigenic potential and terminally differentiate (20). The combination of IFN-beta plus MEZ reprograms metastatic MM cells to undergo a loss of cancerous potential and to terminally differentiate.

In genetic therapy for MM, mda-7 has an important role and study results support future applications of mda-7 in gene-based therapy for MM (20). Intracellular MDA-7 protein is found in most of the melanocyte/melanoma cell lines and its se-

cretion is readily detected following Ad.mda-7 infection of both melanocytes and melanoma cells. Downregulation of MDA-7 protein expression in primary melanomas facilitates progression to invasive and metastatic stages, supporting development of Ad-mda7 as gene therapy for advanced melanoma (21).

Genetic immunization has become attractive for the development of melanoma vaccines, and a number of antigens recognized by cellular components of the immune system have been identified at the molecular level (22). These melanoma antigens include normal cellular proteins such as the tyrosinase family of enzymes involved in melanin synthesis, expressed in melanocytes and melanoma cells. The mechanisms maintaining peripheral tolerance to self-Ags present a major obstacle for the development of antigen-specific melanoma vaccines, presumably because self-Ags are not able to stimulate CD4 T-helper response (22). Analysis of melanosomal enzyme tyrosinase-related protein 2 (TRP2) expressed by melanocytes and most melanoma cells and genetic immunization of mice with cDNA encoding a fusion protein between enhanced green fluorescent protein (EGFP) and autologous murine TRP2 (EGFP. mTRP2) resulted in stimulation of TRP2-reactive T cell. The immunization with EGFP.mTRP2 effectively protected mice against metastatic growth of B16 melanoma. The study results showed that tumor vaccines consisting of self-antigens linked to immunogenic helper sequences could be successfully applied to immunotherapy for melanoma (22).

There are also other gene therapy mechanisms based on cell tumor, which may be used in the prevention of metastatic melanoma. In tumor cell invasion, the spread of MM and the degradation of extracellular matrix proteins matrix metalloproteinases are involved, such as tissue inhibitor of metalloproteinase-1 (TIPM-1) used as gene therapy, which prevents tumor cell invasion and metastasis by preserving extracellular matrix integrity (23). Experimental examination on mice showed that pulmonary metastases were significantly reduced after 4 weeks of TIMP-1 treatment (23).

MOLECULAR BIOLOGY

Convergent evidence from genetic linkage studies of melanoma-prone families and molecular analyses of regulators of cell cycle checkpoints have led to the discovery of a major gene for MM and to elucidation of its probable mechanism of action (24). There exists a large body of literature characterizing p16 deletions and mutations in a wide range of cancers and in familial melanoma. Alterations in the p16 gene are very common in cultured melanoma cells, the rate averaging approximately 70% across numerous studies (24). Cell progression through the G1 phase of the cell division cycle is dependent on molecular complexes formed from the catalytic subunit, CDK4 (cyclin-dependent kinase 4), and the regulatory subunit, cyclin D1.

Approximately 10% of MM patients have family histories of the disease, and approximately 20% of families with a predisposition to melanoma have germline mutations in the CDKN2A tumor-suppressor gene, also known as p16, p16 INK4a, or MTS1, which encodes a cell-cycle regulator that inhibits activities of protein kinases cdk4 and cak6 (25). Some authors examined experimental models of expression of different molecules on MM cells which promote tumor activation and vascularization. Membrane-type metalloproteinase-1 (MT1-MMP) is a transmembrane metalloproteinase overexpressed in tumors, which plays a major role in the first step of pro-MMP-2 activation, leading to the generation of an intermediate 62 kDa species (26). The second step of MMP-2 activation that yields the mature form is less understood and could involve an autocatalytic process and/or the activity of the plasminogen/plasmin system. It has been revealed on an experimental model that MT1-MMP expression by tumor cells promotes tumor vascularization. Different cytokines may be involved in tumor activities, such as hepatocyte growth factor/scatter factor (HGF/SF), a multifunctional cytokine capable of eliciting mitogenic, mutagenic and morphogenetic activities in tumor development and metastasis. Members of the mitogen-activated protein kinase (MAPK) superfamily, including p38 kinase and stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK), play a central role in mediating cellular response to environmental stress, growth factors and cytokines (27). Notably, the p38 kinase-specific inhibitor was able to block melanoma cell proliferation but not motility. Recio *et al*. have shown that the ATF-2 transcription factor becomes activated by HGF/SF through p38 MAPK and SAPK/JNK. Moreover, the p38-ATF-2 pathway can help mediate proliferation signals in tumor cells through transcription activation of key cell cycle regulators (27).

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Numerous studies have demonstrated the expression of surviving, 16.5 kDa protein, in MM, which inhibits the two early apoptotic enzymes caspase-3 and caspase-7, and thus prevents programmed cell death (28). Surviving may act simultaneously with the bel-2 family proteins, but has a different apoptosis inhibitory mechanism. As a positive correlation between surviving expression and tumor grading, and its indication of tumor recurrence after resection or chemotherapy, it could be potentially diagnostic and a prognostic marker in monitoring MM patients (28).

It is also important to mention the role of B-raf oncogene (BRAF) mutations in primary cutaneous MM. Namely, somatic mutations of BRAF kinase, a component of the Ras-mitogen-activated protein/extracellular signal-regulated kinase kinase-mitogen-activated protein kinase pathway, are frequently reported (>65%) in MM and nevi, with a relation to tumor progression and an effect on disease outcome (29).

CHEMOTHERAPY

Numerous chemotherapeutic agents have shown activity in the treatment of metastatic MM. The most widely used chemotherapeutic is dacarbazine (dimethyl triazene imidazole carboxamide; DTIC), especially in previously untreated patients and those with nonvisceral disease (MM of the skin, soft tissue or lymph nodes), with a response rate of 15%-20% (30). However, a very modest effect was recorded in advanced MM. Other chemotherapeutics that have been tried in the treatment of stage III MM are tamoxifen, thymopectin, carmustine (BCNU), cisplatin, bleomycin, yemustin, vincristine, carboplatin, and fotemustine (31-34). They can be combined with IL-2 and IFN-alfa-chemoimmunotherapy. Some researchers have looked for ways to make the existing chemotherapeutic agents more effective.

Different plasma membrane enzymes may have an important role in cellular thiols expressed in tumor cells. Thus, the [gamma]-glutamyl transpeptidase (GGT) enzyme is often expressed in MM and other malignant tumors, although its levels may vary widely among different tumors or cells of the tumor, but plays a crucial role in cellular handling of thiols (35). Thiols are recognized factors in the modulation of cell sensitivity to platinum-based anticancer drugs, and thiol redox status can affect important functions both intracellularly and extracellularly.

COMBINED THERAPY (BIOCHEMOTHERAPY)

There are many experimental trials using combined therapy for MM including chemotherapy (DTIC) and biologic therapy (IL-2, IFN-alfa, IFN-gamma). High-dose IFN-alfa-2b is a pleiotropic cytokine with a potential antitumor effect, and is the only Food and Drug Administration approved adjuvant treatment for MM patients who are at a high risk of recurrence (36). Surgically resected melanoma patients with high-risk features commonly receive adjuvant therapy with IFN-alfa-2b combined with radiotherapy, in spite of its high toxicity. A retrospective chart review study of patients treated with IFN during radiotherapy or within one month of its completion elucidated the potential for enhancement of radiation toxicity by IFN (37). Although IFN may have an impact on disease-free survival, the longterm impact is uncertain, and a recent systematic review of randomized controlled trials of MM demonstrated no clear benefit on overall survival (38-40). IFN-gamma is another pleiotropic cytokine with antitumor activities, including cell growth inhibition, natural killer (NK) cell and CTL activation, and angiogenesis inhibitory activity. These activities are thought to be involved in its antitumor activity. Thus, IFN-gamma prevents B16 MM experimental metastasis by directly inhibiting cell growth although antitumor host functions were noted. There is circumstantial evidence that points to a potentially harmful effect of IFN-alfa-2b on platelet function and severe impairment of platelet aggregation, which appears to be dose-dependent and cumulative dose-dependent. It is supposed that the antiaggregation activity may be the mechanism by which IFN delays, reduces, or prevents the formation of melanoma metastases.

TNF, IFN-gamma, GM-CSF and IL-1 have a strong modulatory and immunoproliferative action, thus being potentially immunotherapeutic. Cytokine therapy is associated with significant reversible toxicity; however, it results in a high degree of tumor destruction. There has been a recent emphasis on the investigation of immunotherapy approaches to melanoma treatment. Although the benefit of these approaches has been uncertain so far, further investigation in this area as well as combination immunotherapy may prove valuable. The role of biochemotherapy (combined chemotherapy) remains to be seen as it has so far showed good response rates but not much impact on overall survival.

CONCLUSION

The many successful results obtained in MM patients open highly interesting prospects of great importance for melanoma development, progression and management. The experimental results may influence the molecule expression in normal melanocytes and nevi, and increasingly reduce expression in radial growth phase, vertical growth phase and metastatic melanoma. Molecular therapeutics and vaccine development will be an important focus for the next decade as is the ever challenging task of identifying a systemic adjuvant therapy regimen for high-risk melanoma. The results recorded in many studies show that tumor vaccines consisting of self-antigens linked to immunogenic helper sequences could be successfully applied to immunotherapy for MM and provide a scientific basis for the translation of this strategy into future clinical investigations.

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